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A New Technique for the Cytogenetic Study of Human Oocytes

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=Abstract=**A technique for making good quality chromosome preparations from human oocytes is described. Analyzable good quality chromosomes were present in human oocytes that were hypotonically treated and fixed.**

Ninety-eight oocytes were processed. Chromosome analysis could be performed on 80 (82%) oocytes. Seven oocytes showed an absence of chromosomes, mainly due to degeneration. Chromosome analysis could not be performed on 11 oocytes because the chromosome number could not be determined. Of 80 analyzable preparations, a 63% (50) incidence of aneuploidy with/without premature chromosome condensation was observed in the human oocytes that failed to fertilize in vitro with 3:4 ratios of hypo and hyperhaploidy.

Our method presented in this study gives an excellent scattering of nuclei and metaphase plates in one layer as well as a good spreading of chromosomes. It may be applied to all stages of human oocytes in IVF-ET programs. This method can also be useful in pre-embryo biopsy and in secondhand quality control for an IVF-ET program. This may help resolve the present limitations of oocyte chromosome preparation and analysis, and therefore lead to further improvements in germ cell cytogenetics.

Key Words: *Oocyte chromosome, Inseminated human oocytes, Aneuploidy, Premature chromosome condensation*

INTRODUCTION

Chromosomal abnormalities and abnormal embryonic development are the major causes of preimplantation embryonic death, implantation failure, or spontaneous abortion. These abnormalities may arise, not only after fertilization, but even earlier, during meiotic maturation of human oocytes.

Despite the fact that several thousand children have been born as a result of in vitro fertilization (IVF) and embryo transfer (ET), it is clear that the pregnancy rate 15% to 25% (Bongso *et al*. 1988), is not satisfactory. This high failure rate may be related to chromosomal aberrations of gametes or culture conditions in vitro.

The technical problems encountered in obtaining mature human oocytes and abnormal human embryos for the purpose of chromosome studies have been considerable. Analyses of human oocyte chromosomes have been carried out on materials obtained from ovariectomy or biopsy, but the quality of the preparations made even the numerical assessments a difficult task. Although the IVF technique has allowed new access to human oocytes, the interpretation of the few published accounts of chromosome preparations from oocytes and fertilized ova has not always been clear (Michelmann & Mettler 1985; Zenzes *et al*. 1985). Thus, improved techniques and systematic studies on

human oocytes and early cleavage stages have been lacking.

The increasing use of IVF in human infertility cases emphasizes the interest in karyotyping human oocytes. However, no reliable method for setting up chromosome preparations of human oocytes has yet been described. The air-drying technique originally developed for mouse eggs was initially suggested to be applicable to the eggs of all mammals, up to the morula stage (Tarkowsky 1966). The success rates achieved with such preparations have been generally low (McGaughey & Chang 1969; Gosden 1973; Wramsby *et al*. 1982). Wramsby & Liedholm (1984) used a gradual fixation technique involving many steps to overcome this problem in human oocytes and claimed that the technique allows chromosome preparation of mammalian oocytes without disruption of the cytoplasmic membranes. They also reported that it is nev-er possible to guarantee that chromosomes are not being lost when dealing with single cells.

This paper presents a good technique for human oocyte chromosome analysis and highlights in the procedure that which will prevent the artefactual loss of chromosomes.

MATERIALS AND METHODS

From October 1989 until May 1990, unfertilized oocytes were obtained from our IVF-ET program. Ninety-eight morphologically unfertilized oocytes were cytogenetically studied. The material for this study came from 44 patients.

Follicular stimulations were achieved with FSH /hMG with/without GnRH-a (long protocol). Oocyte retrieval was performed by ultrasound guided aspiration 36 hrs. after injection of hCG.

Following a preinsemination incubation of the oocytes for a period of about 6 hrs., 50,000 motile sperms per oocyte were used for insemination. About 44 hours postinsemination, the normal embryos were replaced in the patient's uterus. Cytogenetic analysis was attempted in oocytes which remained unfertilized in rejected oocytes.

Collected oocytes were incubated in culture medium (Ham's F-10 + 15% human fetal cord serum) containing colcemid (0.4 μg/ml) for 6 hrs., in order to arrest the oocytes at metaphase. The oocytes were freed from the last cumulus cells mechanically using a flame drawn pasteur pipette (micropipette) connected to a mouth tube. Whereafter the oocytes were transferred to 0.5 ml droplet of 0.5% sodium citrate for 30 minutes of hypotonic treatment. During this time, fresh fixatives were mixed: fixative A consisting of distilled water, glacial acetic acid, and methanol (5 : 1 : 4) and fixative B consisting of methanol and glacial acetic acid (3 : 1). The use of enzymatic treatments of the cumulus cell and zona pellucida is not necessary.

Each oocyte was transferred into a mouth-controlled micropipette which was filled with fixative A. After 30 seconds, with the help of a mouth-controlled pipette, a microdrop (approximately 20 μl) of fixative A together with oocytes (several oocytes can be treated together) was placed in the middle of a grease-free glass slide and then slightly dried. Four drops of fixative B were expelled onto the oocyte, and then the slide preparations were fixed in fixative B for more than 24 hours. Fixed preparations were stained with a 10% Giemsa in a phosphate buffer, pH 6.8 for 10 minutes.

RESULTS

Analyzable good quality chromosomes were present in human oocytes that were hypotonically treated and fixed. By taking extreme care and caution during each step, it was possible to retain the ooplasmic matrix as a circular lightly stained area. Chromosomes, of good quality, were located within the boundaries of this matrix and were sufficiently scattered for counting (Fig. 1).

Table 1 shows the results of the cytogenetic analysis of the oocytes. Ninety-eight oocytes were processed. Chromosome analysis could be per-

Table 1. Chromosome analysis of 98 unfertilized oocytes after IVF

Oocytes Fixed	98
Oocytes Analyzable	80 (82%)
Oocytes Nonanalyzable	18 (18%)
Chromosome clumped	11
No chromosomes	7

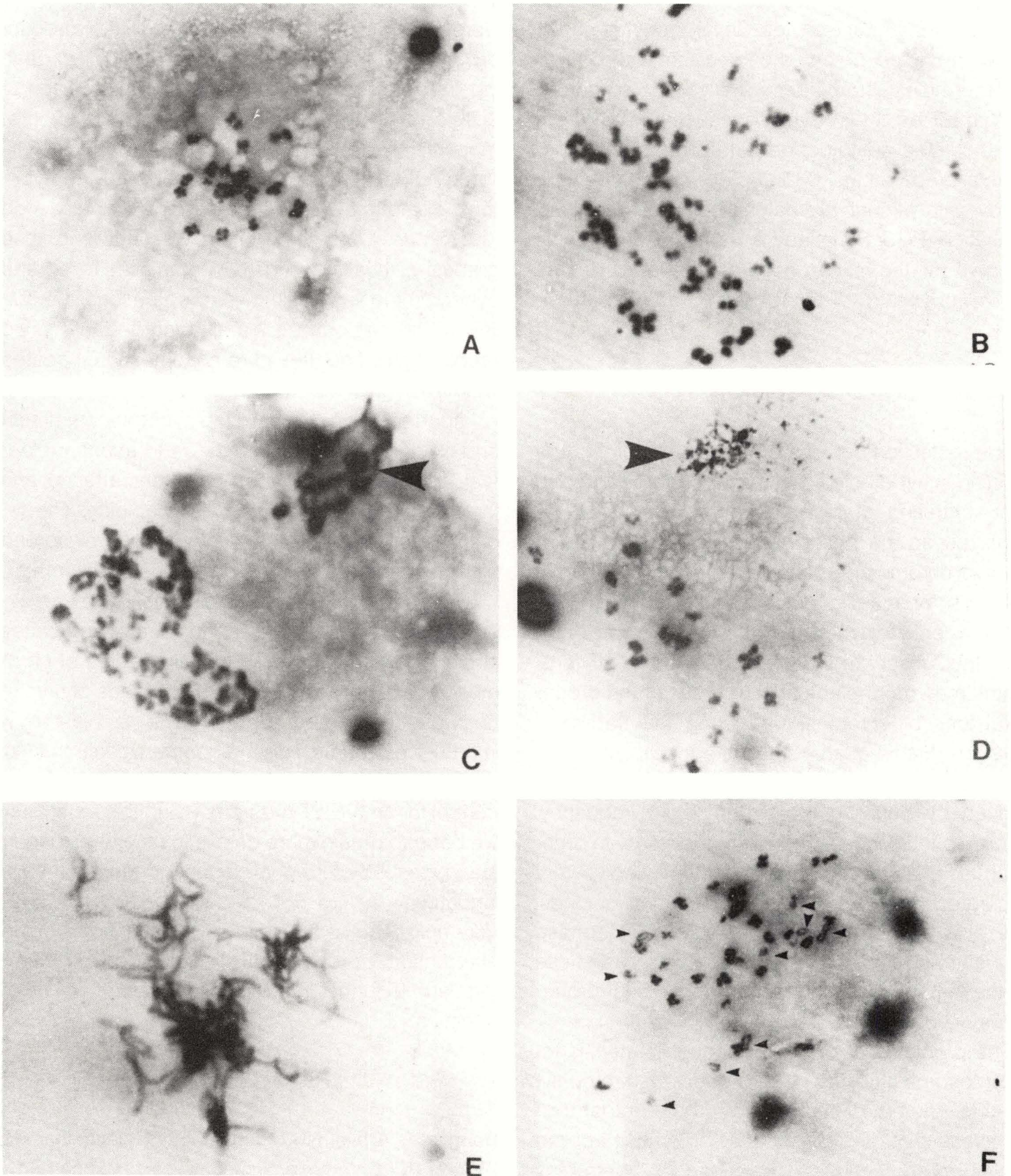

Fig. 1. Giemsa-stained chromosome complements from unfertilized oocytes x1,000. (A) Unfertilized oocyte chromosomes with metaphase II of meiosis are quite distinct from the 23 chromosomes. (B) A diploid set chromosomes of metaphase II from unfertilized oocyte. (C) Oocyte chromosomes with a diploid set of metaphase II chromosomes and prematurely condensed chromosomes. The sperm chromosomes (arrow) are single and long chromatid (G_1-PCC). (D) Oocyte chromosomes with a metaphase II chromosomes and prematurely condensed chromosomes. The sperm chromosomes (arrow) are 'pulverized' S-PCC. (E) Unfertilized oocyte chromosomes with prematurely condensed sperm chromosomes (G_1-PCC only). There are no maternal metaphase II chromosomes. (F) Oocyte chromosomes with prematurely condensed sperm chromosomes (arrows) and maternal metaphase II chromosomes. The prematurely condensed chromosomes are still longer than metaphase II chromosomes.

formed on 80(82%) oocytes. Seven oocytes showed an absence of chromosomes, mainly due to degeneration. Chromosome analysis could not be performed on 11 oocytes because the chromosome number could not be determined. Of 80 analyzable preparations, 63% (50) incidence of aneuploidy with/without premature chromosome condensation (PCC; Schmiady & Kentenich 1989) was observed in the human oocytes that failed to fertilize in vitro with 3 : 4 ratios of hypo and hyperhaploidy.

DISCUSSION

The restrictions of loss of oocytes by premature bursting in hypotonic solutions, poor spreading and over-scattering of chromosomes have been recognized as the major handicaps of oocyte and embryo chromosome analysis using the traditional methods for making preparations. Further, it has been recently claimed that the hypohaploid complements observed in human oocytes in IVF programs may be artefactual as a result of the procedural loss of chromosomes using the traditional methods (Martin *et al*. 1986). As a result, conservative estimates of the frequency of aneuploidy were derived by doubling the hyperhaploid frequency. A reliable technique is therefore necessary in order to obtain analyzable preparations that will give very accurate chromosome counts in human oocytes. It is also necessary for good quality metaphase for identifying structural rearrangements via the use of the established chromosome banding procedures.

The problems of obtaining enough materials for chromosome studies on human oocytes are considerable. Therefore, the reliability of the preparation technique is of great importance. When we applied the air-drying technique described by Tarkowsky (1966) on human oocytes, 12 out of 30 preparations could be analyzed. When we also applied the gradual fixation method reported by Wramsby & Liedholm (1984), only about 50% of preparations could be analyzed. But our own cytogenetic technique was found to give numerical analyzability to 82% of preparations in this study. Our technique described in this study whereby chromosomes were retained within the ooplasmic matrix produced 3:4 ratios of hypo and hyperhaploidy suggesting that errors occurred during oogenesis and/or culture rather than from technical artefactual chromosome loss. The explanation for the higher efficiency of this technique is probably that the hypotonic treatment was performed for a longer period (30 minutes) and the fixation was accomplished gradually, thus allowing the cytoplasm to spread evenly. In the vast majority of preparations, the outline of the cell can be clearly identified in stained preparations and the chromosomes are located well within this borderline.

Important suggestions in the hypotonic treatment and fixation are highlighted so as to avoid artefactual loss of chromosomes by overscattering and thereby interpreting a false hypohaploidy. The technique provides for the retention of well-spread chromosomes within the boundaries of this matrix.

Our method presented in this study gives an excellent scattering of nuclei and metaphase plates in one layer as well as a good spreading of chromosomes. It may be applied to all stages of human oocytes from preovulatory oocyte to blastocyst in IVF-ET programs. This method can also be useful in pre-embryo biopsy and in secondhand quality control for an IVF-ET program. Using this technique, we hope to obtain more details to help us to understand the full picture of abnormal oogenesis and/or incomplete in-vitro oocyte culture conditions. This may help resolve the present limitations of oocyte chromosome preparation and analysis, and therefore lead to further improvements in germ cell cytogenetics.

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=국 문 초 록=

인간 난자의 세포유전학적 연구를 위한 염색체 분석법의 개발

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방명걸 · 오선경 · 황도영 · 문신용 · 이진용 · 장윤석 · 최규완*

1978년 Louise Brown이 자연배란법을 이용한 체외수정으로 태어난 후 체외수정은 전세계 불임부부에 희망을 안겨 주는 계기가 되었다. 그 후 체외수정의 성공률을 높이는 방법으로 과배란 기술의 발달과 배양조건의 점진적인 개선을 통하여 최근에 이르러서는 15-25%의 임신성공률을 이루게 되었다. 그러나 상대적으로 높은 실패율과 높은 자연유산율은 현재 체외수정의 큰 장애요인으로 대두되고 있는 실정이다. 그 후 여러 연구를 통해 이러한 실패요인의 많은 부분이 과배란 유도와 불완전한 배양조건에 의한 난자의 염색체 이상에 기인한다고 밝혀졌다. 그러나 이와 같은 인간 생식세포의 염색체 이상을 분석하는 기술상의 난점으로 이에 대한 연구가 극히 미진한 상태에 머물고 있다.

본 연구는 체외수정 프로그램 시행중 배아이식 대상에서 제외된 미수정 난자를 이용하여 난자의 염색체 이상을 분석할 수 있는 새로운 기술을 개발하고, 이 방법을 이용하여 인간 난자의 염색체 분석을 하고자 시행되었다. 처리된 난자 98개 중 80개(82%)에서 염색체를 확인할 수 있었다. 분석결과, 염색체수의 이상 및 두 생식세포(정자, 난자)의 비동시성(allocycle)로 인한 미성숙 염색체 응축(premature chromosome condensation) 등의 염색체 이상을 분석하기에 좋은 방법으로 사료된다.